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Research

A neuronal-specific differentiation protein that directly modulates retinoid receptor transcriptional activation

Kenneth W Henry II, Michael L Spencer, Maria Theodosiou, Dingyuan Lou and Daniel J Noonan*

Address: Department of Molecular and Cellular Biochemistry, University of Kentucky, 800 Rose Street, Lexington, KY 40536, USA

Email: Kenneth W Henry II - khenry@sequenom.com; Michael L Spencer - mspen1@pop.uky.edu; Maria Theodosiou - mtheo2@pop.uky.edu; Dingyuan Lou - dlou@pop.uky.edu; Daniel J Noonan* - dnoonan@pop.uky.edu

* Corresponding author

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Abstract

Background: The specificity of a nuclear receptor's ability to modulate gene expression resides in its ability to bind a specific lipophilic ligand, associate with specific dimerization partners and bind specific DNA sequences in the promoter regions of genes. This sequence of events appears to be the basis for targeting an additional regulatory complex composed of a variety of protein and RNA components that deliver signals for facilitation or inhibition of the RNA polymerase complex. Characterization of the tissue and cell-specific components of these coregulatory complexes appear to be integral to our understanding of nuclear receptor regulation of transcription.

Results: A novel yeast screen sensitive to retinoid-X receptor (RXR) transcriptional activation resulted in the isolation of the rat homologue of the mouse NPDC-1 gene. NPDC-1 has been shown to be involved in the control of neural cell proliferation and differentiation, possibly through interactions with the cell cycle promoting transcription factor E2F-1. Although the amino acid sequence of NPDC-1 is highly conserved between mouse, rat and human homologues, their tissue specific expression was seen to vary. A potential for direct protein:protein interaction between NPDC-1, RXR and retinoic acid receptor beta (RARβ) was observed in vitro and NPDC-1 facilitated RXR homodimer and RAR-RXR heterodimer DNA binding in vitro. Expression of NPDC-1 was also observed to repress transcription mediated by retinoid receptors as well as by several other nuclear receptor family members, although not in a universal manner.

Conclusions: These results suggest that NPDC-1, through direct interaction with retinoid receptors, functions to enhance the transcription complex formation and DNA binding function of retinoid receptors, but ultimately repress retinoid receptor-mediated gene expression. As with NPDC-1, retinoids and their receptors have been implicated in brain development and these data provide a point of convergence for NPDC-1 and retinoid mediation of neuronal differentiation.

Background

Retinoids are a class of compounds that demonstrate a profound effect on cellular differentiation and proliferation [1–4]. The physiologically active retinoid metabolite, all-trans retinoic acid (atRA) regulates gene expression by complexing with members of the steroid/hormone family of nuclear receptors. Two groups of nuclear receptors have been shown to mediate retinoid signaling. The three

isotypes (α, β and γ) of retinoic acid receptor (RAR) bind atRA in acquiring a transcriptionally active state [3,5,6]. A second group of receptors, called the retinoid-X receptors (RXRs; isotypes α, β and γ), bind to an atRA isomer, 9-cis retinoic acid (9cRA), and can activate transcription as a homodimer complex [6–8]. However, the predominant physiological function of RXRs may be as an obligate heterodimer partner for a number of other nuclear receptors including the thyroid hormone receptors, the peroxisome proliferator activated receptors, the vitamin D receptor as well as the RARs [9–11].

Steroid/hormone nuclear receptors regulate the expression of specific target genes by binding to cis-acting DNA sequences called hormone response elements (HREs). RAR/RXR heterodimers preferentially bind HREs categorized as direct repeats of the half-site consensus sequence AGGTCA or AGTTCA separated by five or two nucleotides, referred to as a DR5 and DR2 respectively. One of the first and most potent RAR HRE identified was the DR5 motif in the promoter of the RARβ gene itself [12].

Retinoid induced transcriptional activation in mammalian cells is influenced by the cell cycle regulator E2F-1 [13]. The E2F-1 transcription factor is part of a multi-protein family that regulates the expression of gene products needed for the initiation and completion of DNA synthesis [14,15]. E2F-1 and other members of this family share the ability to induce the transition from G1 to S phase of the cell cycle. E2F dependent transcription is thought to be dominantly regulated by interactions with the retinoblastoma (Rb) gene product and other Rb-related proteins (reviewed in [16]). It has been demonstrated that co-expression of E2F-1 inhibits atRA induced transcription from a RARβ response element driven reporter plasmid [13]. However, the E2F-1 protein could not be demonstrated to directly bind to the RARs or the βRE, suggesting an interaction with a common accessory factor or factors.

The mechanism of steroid/hormone nuclear receptor function includes interaction with a number of recently identified co-regulators [17–21]. The yeast two-hybrid system and far-western blotting have been used to identify several proteins that associate with nuclear receptors in a ligand-dependent manner. These include the co-repressors SMRT and N-CoR, the coactivators SRC-1 and RIP-140 and the integrator proteins p300 and CBP. Recently, a short conserved amino acid sequence motif (LXXLL) has been reported to be necessary and sufficient to mediate the binding of RIP-140, SRC-1 and CBP to liganded nuclear receptors [22,23]. For the most part, these co-regulators have not been shown to be specifically expressed with respect to receptor function and usually demonstrate a significant degree of interaction overlap between various nuclear receptors. RXRs are promiscuous in association with multiple nuclear receptors and therefore would be an excellent target by which co-regulator function could effect sweeping changes in transcriptional regulation associated with complex cellular processes such as differentiation.

We and others have shown that it is possible to reconstitute RXR transcriptional function in yeast [24–26]. In this study, we attempted to identify, from randomly expressed rat cDNA sequences, proteins that positively influence RXR mediated transcription in yeast. A cDNA sequence was isolated containing an open reading frame (ORF) which coded for a protein that has a very high amino acid identity (94%) with a previously described mouse neural proliferation, differentiation and control protein (NPDC-1) [27]. More recently, a human isoform has been identified sharing similar structural feature but differing in its tissue distribution [28]. Mouse NPDC-1 expression has been demonstrated to down-regulate cell proliferation in a reversible fashion and suppress transformation properties when stably transfected into cell lines [27]. A protein-protein interaction has also been shown between mouse NPDC-1 and the cell cycle regulator E2F-1 [29,30]. Alignment of the mouse, rat and human NPDC-1 proteins reveals a conserved LXXLL amino acid sequence proximal to their amino terminus. In this paper we demonstrate that NPDC-1 can physically interact with RXR and RAR, and can repress retinoid signaling in a mammalian cell transcription assay. We also show that NPDC-1 can repress transcription events mediated by retinoid receptors and several other steroid/nuclear receptor family members, can modulate binding of retinoid receptors to their response elements and can inhibit the endogenous activity of an E2F transcriptional reporter plasmid in mammalian cells. Together with previously published information, these data support a hypothetical role for NPDC-1 in neuronal proliferation and differentiation as a corepressor of retinoid receptor and E2F-1 mediated gene transcription events.

Results

Functional Yeast Screen Identifies a RXR Transcription Co-regulator

RXR transcriptional activation was reconstituted in yeast by manipulation of the ***S. cerevisiae*** auxotrophic mutant cell line BJ5409. Yeast transcription reporter plasmids incorporate a HRE into an enhancerless ***gal* 1** promoter controlling the conditional expression of a βGal gene. A RXR expressing, βRE-βGal reporter plasmid yeast transformant was isolated and maintained by nutritional selection. Confirmation of the maintenance of both plasmids was demonstrated by ligand (9cRA) dose dependent induction of βGal expression (Fig. 1A). A third nutritionally selectable plasmid expressing a rat brain cDNA library (pcRB) [31] was then transformed into these yeast and

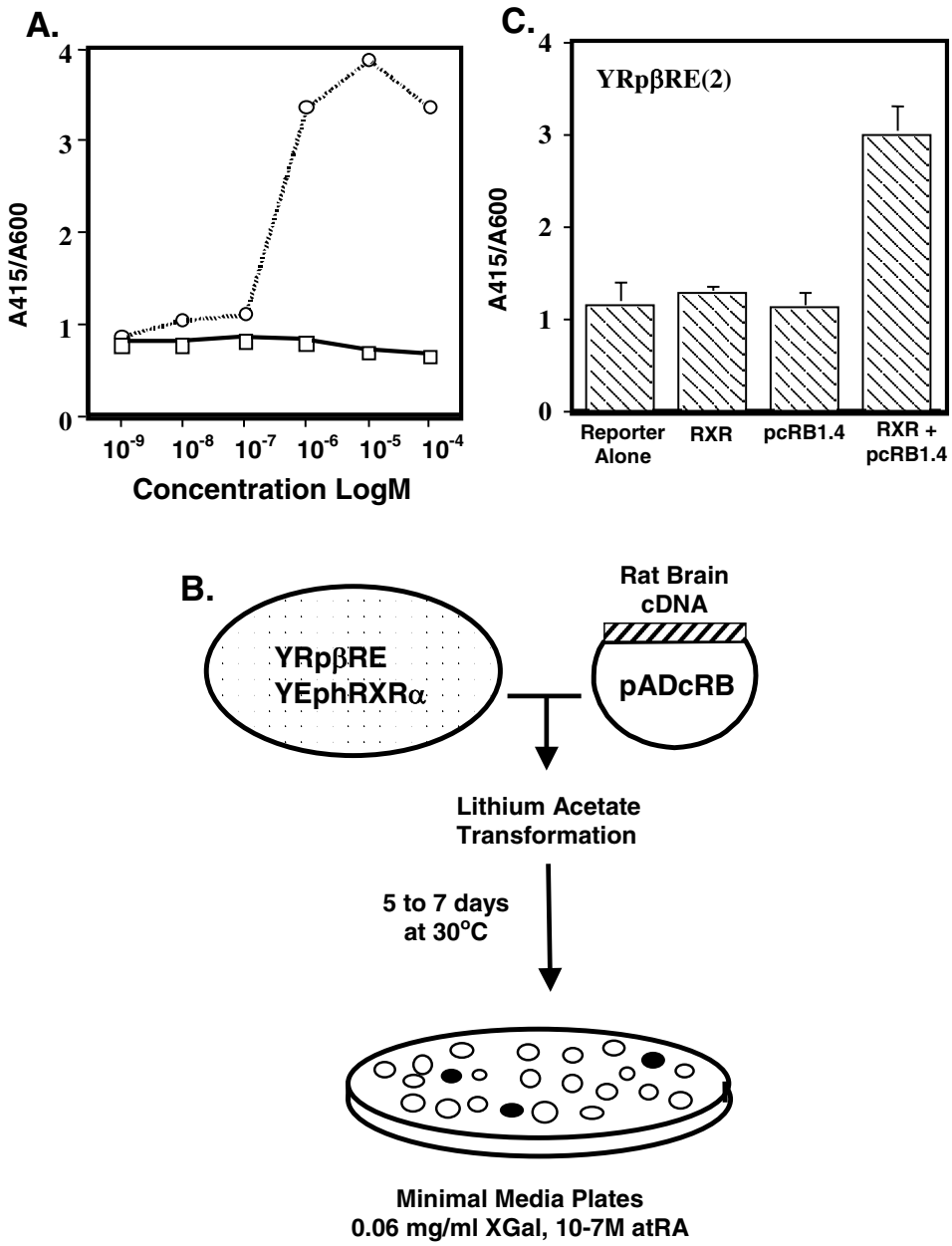

Figure 1

Rat brain cDNA expression library screening in yeast identifies a plasmid containing an approximately 1.4 kb cDNA fragment capable of enhancing RXR transcriptional activation. (A) Yeast cells transformed with the β-galactosidase reporter vector YRpβRE and the yeast expression vector YEpRXRα were exposed to increasing concentrations of 9-cis retinoic acid (9cRA). The βGal activity present in yeast extracts was measured as the colorimetric change of the βGal substrate ONPG (A_{415}) normalized for cell number approximations (A_{600}). The dose response of YRpβRE transformed yeast is also shown (Reporter Alone). Values reported are averages of duplicate data points. (B) An outline of the screening strategy used to identify a RXR transcriptional enhancing cDNA product. Transformed yeast expressing rat brain cDNA products that elevate transcription of the βGal reporter gene result in the formation of blue colonies. (C) BJ5409 yeast cells were transformed with the transcription reporter plasmid YRpβRE(2), co-transformed with either YEpRXRα or pcRB1.4 expression plasmids and YRpβRE(2), or co-transformed with YEpRXRα, pcRB1.4 and YRpβRE(2). The βGal activity present in yeast extracts was measured as in (A). Values reported are averages of multiple data points and error bars reflect standard deviations, n = 3.

plated onto minimal media plates containing the βGal substrate X-GAL (Fig. 1B). Approximately 150,000 transformants were analyzed for the presence of βGal activity (blue colonies).

Several positive colonies were expanded in solution and secondarily tested for βGal activity using the βGal substrate ONPG. The βGal enzymatic activity present in yeast extracts was determined and those cDNA expressing transformants which induced βGal transcription at least 2 fold over RXR/βRE-βGal yeast were expanded in solution and extracts were prepared for plasmid rescue by bacterial transformation [32]. Random bacterial transformants were used to generate plasmid preps. *EcoRI*, *Hind III* restriction enzyme digests generated a DNA fragment pattern which easily identified pcRB insert containing plasmids separate from RXR expression and βRE-βGal reporter plasmids also present in every yeast extract.

A pcRB plasmid containing an insert of approximately 1.4 kb in length was isolated (pcRB1.4). To demonstrate the specific enhancement of RXR mediated transcription versus an enhancement of transcription in general, the pcRB1.4 plasmid was co-transformed with and without RXR expression plasmids. In these experiments a reporter plasmid with two copies of the βRE incorporated into the βGal reporter plasmid was used to increase the sensitivity of the assay. RXR transcription was enhanced by pcRB1.4 two fold over RXR expression alone, but pcRB1.4 did not enhance transcription when co-transformed with the reporter plasmid alone (Fig. 1C). These results demonstrated that the pcRB1.4 plasmid was autonomously capable of specifically enhancing RXR mediated transcription.

DNA Sequencing Reveals the RXR Transcription Coregulator to be NPDC-1

The entire pcRB1.4 insert and sub-fragments were subcloned into pBluescript (Stratagene) for DNA sequencing. A sequence of 1,384 bp was determined. This sequence was aligned with the Genbank database to screen known DNA sequences for homology. A very high homology was displayed between pcRB1.4 and a previously cloned DNA sequence for the mouse gene NPDC-1 [27]. The pcRB1.4 sequence had only one extended ORF coding for a 331 amino acid protein that displayed a 94% identity with the mouse protein NPDC-1 (Fig. 2). As with the mouse sequence, the pcRB1.4 ORF is preceded by a TGA stop codon two triplets upstream of the ATG translation initiation codon. The pcRB1.4 clone also contains a classical polyadenylation signal sequence in the same approximate position as the mouse but ends without a poly-A tail representation. From this information it was concluded that the pcRB1.4 DNA sequence contains the rat homologue of the mouse NPDC-1 gene.

The cDNA for the rNPDC-1 was subsequently used to screen a human embryonic brain λgt11 cDNA library. This screen identified a 2.5 kb cDNA that contained an open reading frame of 325 amino acids and greater than 99% identity with a recently published human homolog for mNPDC-1 [28].

Figure 2 contains an alignment of rNPDC-1 and hNPDC with the published sequence for mNPDC-1. The overall amino acid homology of the NPDC-1 protein between species suggests that these proteins may have a conserved function. Some conserved amino acid motifs may be related to specific function. All three NPDC-1 homologues have a perfectly conserved LXXLL motif (amino acids 15 – 20, Fig. 2), a sequence that has been shown to mediate the interaction of coregulatory proteins with nuclear receptors [22,23].

Northern Analysis of Rat and Human NPDC-1 Tissue Distribution

The rNPDC-1 cDNA sequence was used as a template for nick translation synthesis of a radiolabeled DNA probe. Probe hybridization to a multiple tissue poly-A^+ RNA Northern blot demonstrated the tissue distribution of rNPDC-1 mRNA (Fig. 3). A single abundant mRNA band of approximately 1.5 kb was identified in rat brain. This is consistent with the published expression of NPDC-1 mRNA in mouse brain and suggests that our cloned sequence represents a majority of the NPDC-1 mRNA expressed in rat brain [27]. A band of similar intensity was also present in rat lung. The rat NPDC-1 probe hybridized with three other mRNA transcripts in the lung of approximately 2.0, 5.0 and 6.3 kb in size. The 1.5 kb and 2.0 kb bands were also detectable (in order of decreasing intensity) in kidney, skeletal muscle and heart tissues of the rat. The 1.5 kb and 2.0 kb bands were faintly detectable in the spleen but there was a relatively stronger representation of the 6.3 kb band. The 6.3 kb band was also present in the kidney. The 1.5 kb band was barely detectable in the testis and not at all in liver.

The human NPDC-1 probe identified two mRNA transcripts of approximately 1.5 and 6.3 kb in size in all tissues examined. These data are similar to those reported by Qu et. al. [28] and unlike the rat, the relative abundance of the two transcripts was more consistent in each specific human tissue. As with NPDC-1 mRNA expression in mice and rats, human NPDC-1 mRNA was most abundant in the brain. A considerable amount of NPDC-1 mRNA is present in the heart and pancreas and to a lesser extent in placenta, lung, liver, skeletal muscle and kidney. Since the cloned human cDNA fragment identified here is approximately 2.3 kb in size, has a poly-A tail representation and a TGA codon immediately upstream of the NPDC-1 ORF, it is possible that it represents a portion of the 6.3 kb

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mouse     1 MATPVPPPSPRHLRL LRLLL SGLILGAALNGATARRPDAT 40
rat       1 MATPVPPPSPRHLRL LRLLL SGLILGAALNGATARRPDAP 40
human     1 MATPLPPPSPRHLRL LRLLL SGLVLGAALRGAAAGHPDVA 40
            ****.********** ***** ***.***** **.* .**

mouse    41 TCPGSLDCALKRRAKCPPGAHACGPCLQSFQEDQRGFCVP  80
rat      41 TCPGSLDCALKRRAKCPPGAHACGPCLQSFQEDQRGVCVP  80
human    41 ACPGSLDCALKRRARCPPGAHACGPCLQPFQEDQQGLCVP  80
            .*************.************* *****.* ***
mouse    81 RKHLSSGEGLPQPRLEEEIDSLAQELALKEKEAGHSRLTA 120
rat      81 RKHQSSGEGLPQPRLEEEIDSLARELALKEKEAGHPRLTA 120
human    81 RMRRPPGGGRPQPRLEDEIDFLAQELARKE--SGHS---T 115
            * .   * * ******.*** **.*** **  .**    .
mouse   121 QPLLERAQKLLEPAATLGFSQWGQRLEPGLPSTHGTSSPI 160
rat     121 QPLPEASQKLLEPAATLGFSQWGQQLEPGLPSTHGTSSPT 160
human   116 PPLPKDRQRLPEP-ATLGFSARGQGLELGLPSTPGTPTPT 154
             **    *.* ** ******  ** ** ***** ** .*
mouse   161 PHTSLSSRASSGPVQMSPLEPQGRHGNGLTLVLILAFCLA 200
rat     161 PHTSLSARASSGPVQMSPLEPQGR-GNGLALVLILAFCLA 199
human   155 PHTSLGSPVSSDPVHMSPLEPRGGQGDGLALVLILAFCVA 194
            ***** .  ** **.******.*  * **.********.*
mouse   201 SSAALAVAALCWCRLQREIRLTQKADYAATAKGPTSPSTP 240
rat     200 STAALAVAALCWCRLQREIRLTQKADYTATAKGPTSPTTP 239
human   195 GAAALSVASLCWCRLQREIRLTQKADY-ATAKAPGSPAAP 233
             .***.**.****************** **** * **..*
mouse   241 RISPGDQRLAHSAEMYHYQHQRQQMLCLERHKEPPKELES 280
rat     240 RISPGDERLAHSAEMYHYQHQRQQMLCLERHKDPPKELES 279
human   234 RISPGDQRLAQSAEMYHYQHQRQQMLCLERHKEPPKELDT 273
            ******.***.**********************.*****..
mouse   281 ASSDEENEDGDFTVYECPGLAPTGEMEVRNPLFDHSTLSA 320
rat     280 ASSDEENEDGDFTVYECPGLAPTGEMEVRNPLFDHSTLSA 319
human   274 ASSDEENEDGDFTVYECPGLAPTGEMEVRNPLFDHAALSA 313
            ***********************************..***
mouse   321 PVPGPHSLPPLQ 332
rat     320 PVPGPHSSPPLQ 331
human   314 PLPAPSSPPALP 325
            *.* * * * *
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Figure 2
Comparative alignment of the predicted amino acid sequence of rat and human NPDC-1 with the published mouse amino acid sequence. The published mouse NPDC-1 amino acid sequence was aligned with cDNA deduced from rat and human amino acid sequences using the Clustal W(1.4) Multiple Sequence Alignment program (MacVector 7.0.1). An asterisk, "*" indicates conserved amino acid identity and a period "." indicates amino acid similarity between the three sequences. A conserved LXXLL motif is indicated by a box.

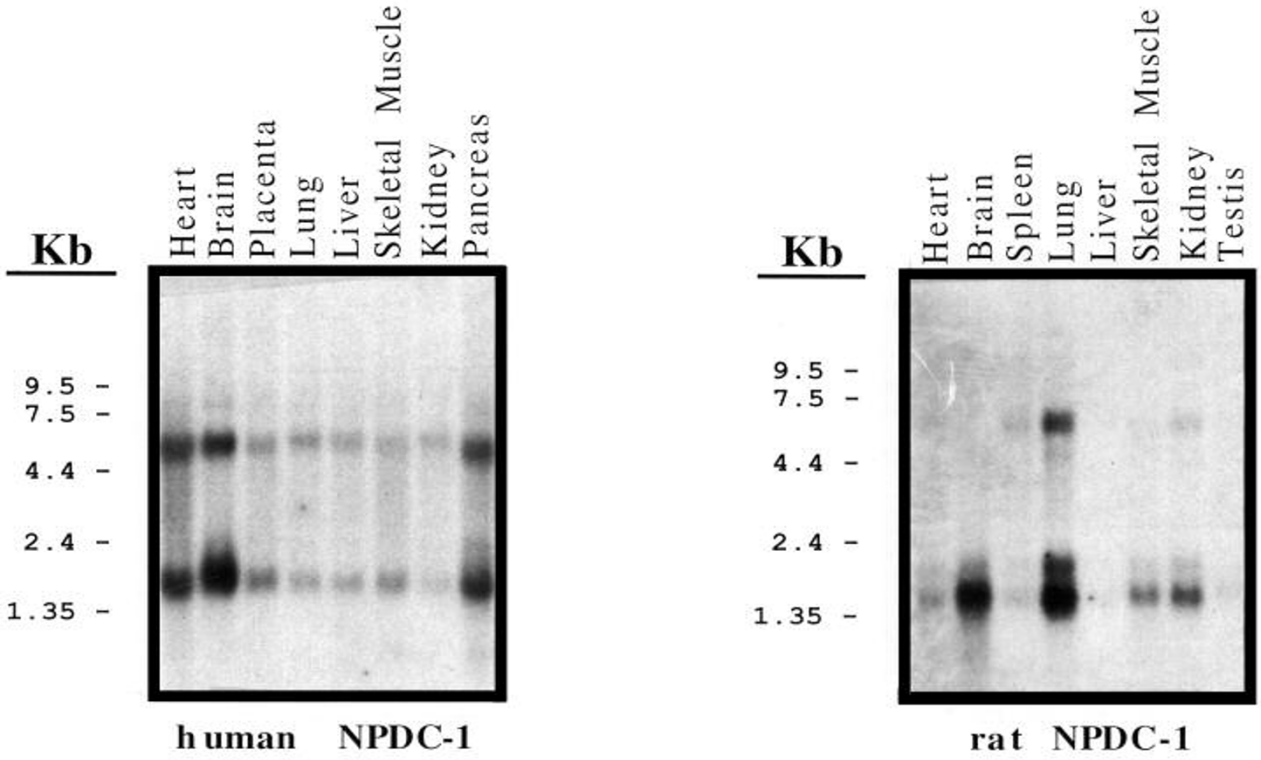

Figure 3

Northern analysis of rat and human NPDC-1 tissue distribution. Approximately 2 μg of poly A^+ RNA from the indicated specific tissues was used to generate rat and human multiple tissues northern blots. Each blot was probed with species-specific cDNA derived radiolabeled DNA probes. The location of RNA size markers are indicated.

transcript minus an unusually extended 5' untranslated region. The 1.5 kb transcript likely represents a splice variant isoform similar in size to the transcript that represents NPDC-1 expression in mice and rats, although the presence of an alternative, very similar gene transcript that is detectable with the human NPDC-1 probe cannot be ruled out.

NPDC-1-RXR and NPDC-1-RAR form Protein-Protein complexes In Vivo

To test if human NPDC-1 could interact with *in vivo* expressed E2F-1, RARβ or RXRα, recombinant Trx-S-NPDC-1 was used to pull-down protein from mammalian cell lysates (Fig. 4). PC12 cells were transfected with pCMV-HA-E2F-1, pRS-hRARβ, or pRS-hRXRα and incubated for 48 hours. Clarified cell lysates were prepared, and incubated for 1 hour at 4°C with a 50% slurry of Trx-S-NPDC beads. Ligands, atRA and 9cRA were diluted to a concentration of 1 micromolar into clarified lysate before the addition of recombinant hNPDC-1. Beads were collected, washed with binding buffer and incubated with S-protein agarose bound to pull out thioredoxin-S-tagged NPDC-1. Bound protein was eluted from beads with electrophoresis sample buffer, run on 12.5% SDS-PAGE gels, Western blotted to nitrocellulose membranes and probed with specific antisera for the presence of HA-tagged E2F-1 (Fig. 4A), RARβ, or RXRα ••••• 4B).

As seen in Fig. 4A recombinant NPDC was able to pull down E2F-1 (Fig. 4A), RXRα and RARβ (Fig. 4B) from PC12 lysates. The interaction with NPDC appeared to be independent of ligand stimulation. In fact, ligand appeared to slightly reduce the ability of NPDC to associate with either RXRα or RARβ. As a binding control we attempted to pull down HA-E2F1 from PC12 lysates. We demonstrate that human NPDC can bind to E2F1 expressed in mammalian cells. Although a mouse NPDC-E2F interaction has been previously demonstrated using in vitro binding and mammalian transcription protocols [29,30], these are the first studies demonstrating a

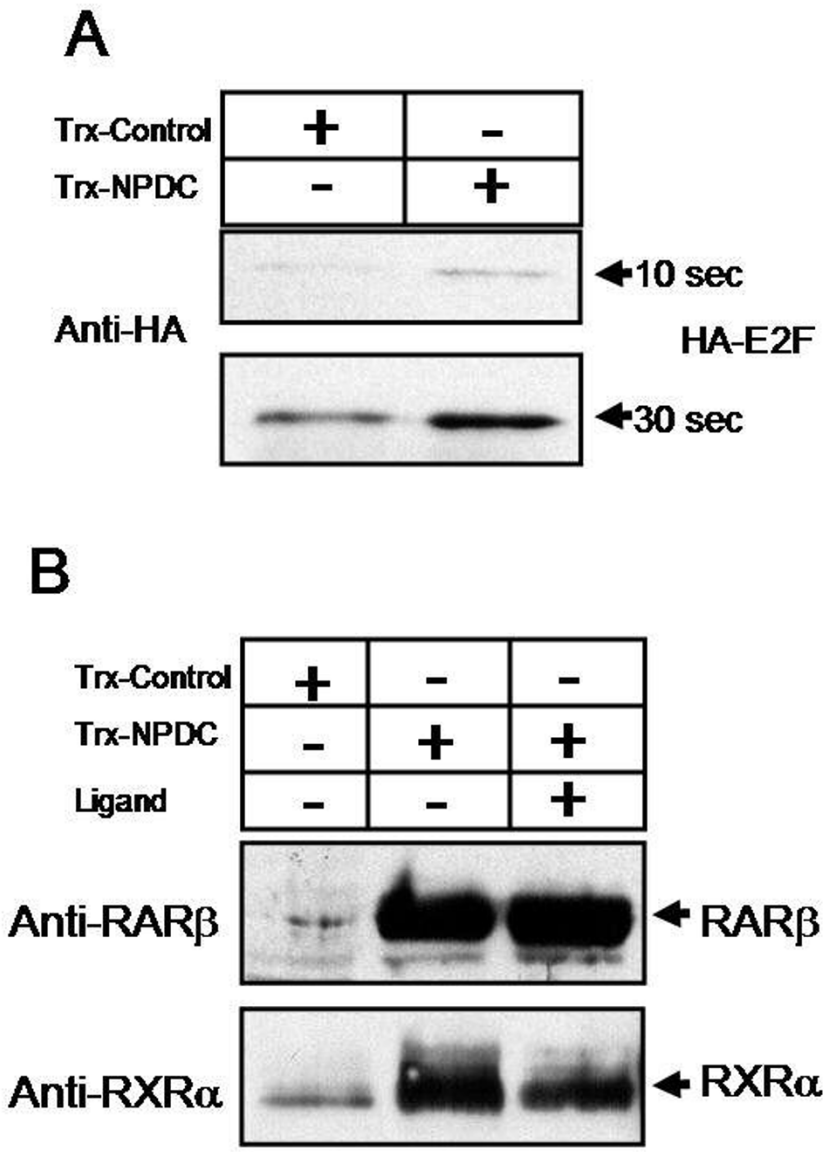

Figure 4

RXR, RAR and E2F-1 proteins form complexes with hNPDC-1 In Vivo PC12 cells were transfected with pCMV-HA-E2F-1, pRS-hRARβ, or pRS-hRXRα and incubated for 48 hours. Subsequently clarified cell lysates were prepared, and incubated for with a 50% slurry of Trx-S-hNPDC beads (bacterially expressed hNPDC fused at its carboxyl terminus with S-peptide and thioredoxin tags and affinity purified by binding to S-protein agarose beads as described in the Methods section). Ligands, atRA and 9cRA were diluted to a concentration of 1 μM into clarified lysate before the addition hNPDC. Beads were collected, washed, bound protein was eluted from beads with 2X SDS electrophoresis sample buffer and proteins were separated on 12.5% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes and probed for the presence of HA-tagged E2F-1, RARβ, or RXRα••••••••••••••

potential for in vivo complex formation between hNPDC-1 and E2F proteins.

NPDC-1 can bind directly to RXR and RXR/RAR complexes

To characterize NPDC-1 and RXR interactions, a bacterial extract of Flag-tagged rNPDC was incubated, in the presence of 9cRA or bacterially expressed hRARβ, with GST-RXR immobilized on glutathione agarose beads. After extensive washing, bound proteins were eluted from the GST-RXR beads, electrophoresed and analyzed by Western blot probing with an anti-flag M2 monoclonal antibody (Fig. 5). This assay demonstrated that agarose bound GST-RXR was able to specifically pull down a protein of the expected size for recombinant rNPDC (F), not found when control agarose bound GST beads were used. These results demonstrate that bacterially expressed hRXRα recombinant protein, in the absence or presence of a homodimer inducing ligand, or in the presence of a hRARβ heterodimer partner, was capable of interacting with NPDC-1.

NPDC-1 Facilitates the In Vitro DNA Binding of RXR Homodimers and RAR/RXR Heterodimers

Ligand induced RXR homodimers and RAR/RXR heterodimers will form in solution and are capable of binding to specific HRE DNA sequences [33–35]. To investigate the impact NPDC-1 has on nuclear receptor DNA binding, recombinant human NPDC-1 was analyzed for its ability to modulate gel mobility shift complexes generated using PC12 cell nuclear extracts and a radiolabeled DR-5 βRARE DNA response element (Fig. 6A). The βRARE generated two major shift complexes in the presence of PC12 extracts. The addition of recombinant hNPDC was observed to substantially enhance the formation of the lower shift complex at the expense of the higher shift complex. NPDC-1 did not form a measurable unique complex with the radiolabeled DNA. Curiously, the enhanced protein:DNA complex formed without a significant change in mobility indicating that NPDC-1 facilitates DNA binding without remaining in strong association with either proteins or DNA.

To measure the direct effect of NPDC-1 on the DNA binding properties of retinoic receptors, RXR and NPDC-1 bacterially expressed protein was tested for binding on a DR-1 RXRE probe (Fig. 6B). As we, and others, have previously observed, RXR bound to the DR-1 probe in the absence of ligand, forming, what appears to be, a homodimer complex (upper band) and a monomer complex (lower band). The addition of NPDC-1 increased both the upper and lower complexes, again without any observable increase in complex mobility. To evaluate the presence or absence of NPDC-1 in these complexes, supershifts were performed with antibodies for RXRα and NPDC-1 (Fig. 6C). Supershift experiments demonstrated that NPDC-1 is not part of the RXR/DR-1 complex and that RXRα is a major component of both lower and upper bands.

hNPDC-1 represses E2F-1 mediated transcription

As demonstrated above and similar to previous reports for mouse NPDC-1 [29,30], human NPDC-1 preferentially binds E2F-1. To determine the functional effect of human NPDC-1 expression on E2F mediated transcription, the E2F response element (E2FRE) found in the promoter region of the dihydrofolate reductase gene was sub-cloned into a transcriptional reporter plasmid [36]. Multiple members of the E2F family of transcription factors have been shown to activate transcription via this type of response element. Transient transfection of the E2FRE reporter plasmid into the human hepatocellular carcinoma cell line HepG2 resulted in a constitutive level of transcriptional activity that was inhibited 65% by the co-transfection of the human NPDC-1 expression plasmid (Fig. 7A). These data with hNPDC-1 are in line with those reported for mNPDC-1 and support a role for this protein in the regulation of molecular events mediated by the human E2F family of transcription factors.

NPDC-1 Functions in Mammalian Cell Transcription Assays

To examine NPDC-1 effects on nuclear receptor mediated transcriptional activation in mammalian cells, the rat NPDC-1 gene was sub-cloned into a cytomegalovirus (CMV) promoter driven mammalian expression plasmid. Transfections of mammalian expression constructs for steroid/nuclear receptor family members hRXRα, hRARβ, rat peroxisome proliferator-activated receptor alpha (rPPARα), human estrogen receptor alpha (ERα) and human vitamin D receptor (hVDR), along with their respective luciferase reporter constructs were analyzed with respect to hNPDC effects on ligand-induced transcriptional activation mediated by these receptors (Fig. 7B). As seen in Figure 7B, NPDC-1 strongly repressed transcription mediated by the retinoid receptors, moderately repressed transcription mediated by PPARα and ERα, and had very little effect on VDR.

NPDC-1 Transcriptional Repression Appears Not to be Through its LXXLL Motif

The above data suggest NPDC-1 functions as a steroid/hormone nuclear receptor corepressor protein [17–21]. Many of the corepressors identified thus far have been shown to mediate their activities through specific interactions between receptors and a short conserved LXXLL amino acid sequence motif found on the repressor proteins [22,23]. Within the amino terminus of the NPDC protein there exists a highly conserved LLRLLL motif (see Fig. 2). To analyze the functional role of this LLRLLL motif

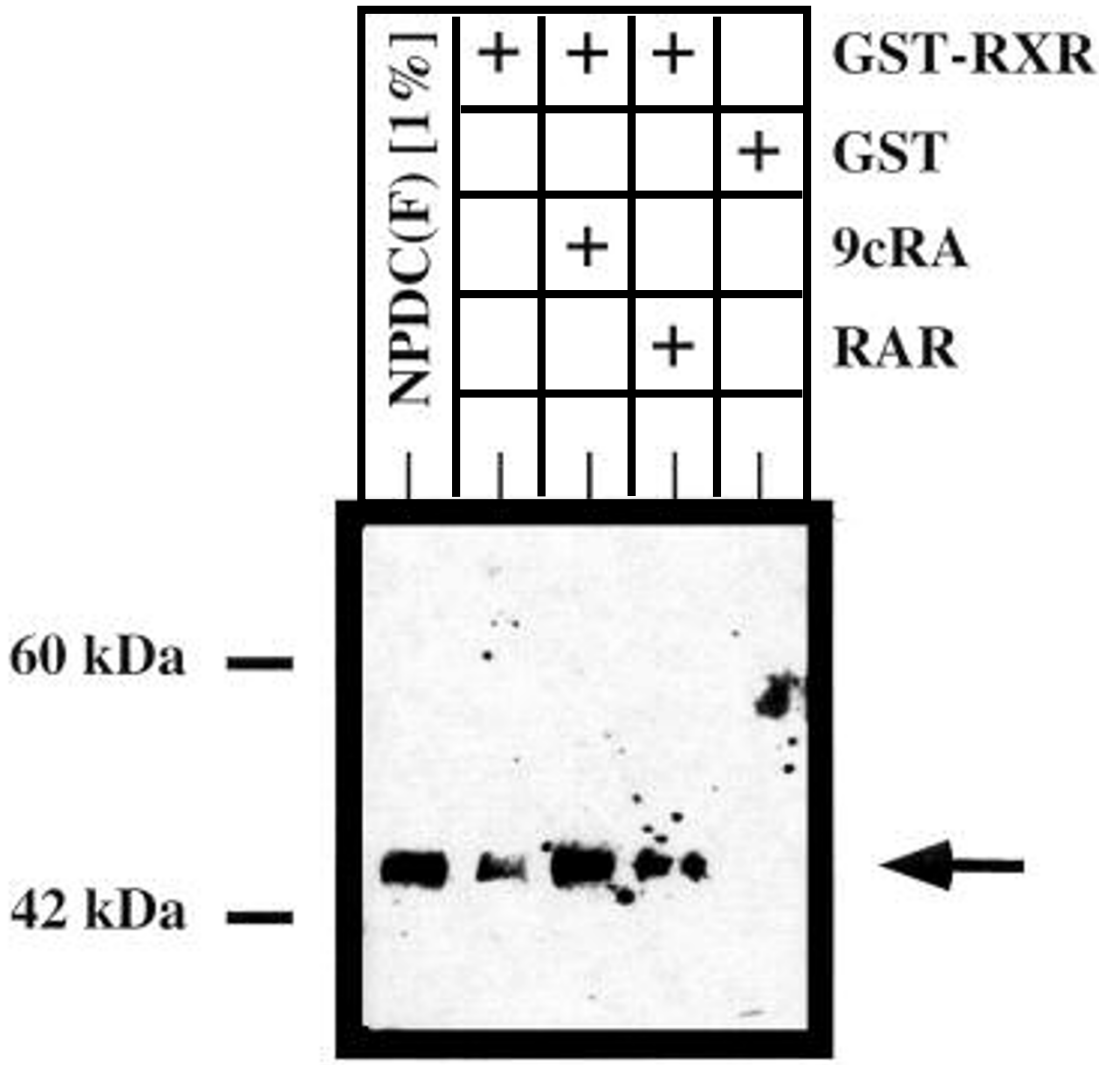

Figure 5
Direct protein-protein interactions between NPDC-1 and RXR. Bacterially expressed flag epitope tagged NPDC-1 [NPDC(F)] (40 μg) was incubated with glutathione-agarose bound GST/human RXRα (GST-RXR) fusion protein in the absence or presence of bacterially expressed human RARγ (RAR) (60 μg) or in the presence of 9-cis retinoic acid (9cRA) (10^{-6} M) as indicated. NPDC(F) was also incubated with glutathione-agarose bound GST. After extensive washing, the retained proteins were resolved by gel electrophoresis and analyzed by immunoblot with anti-flag M2 antibodies. One percent (0.4 μg) of the available NPDC(F) input was also electrophoresed and a unique immunoreactive band of approximately 45 kDa representing NPDC(F) is indicated by an arrow. The position of prestained molecular weight standards are shown.

in transcriptional repression mediated by NPDC-1, a series of mutations in the LLRLLL motif were made that sequentially converted key leucines into alanines. These mutated NPDC-1 constructs were analyzed in the mam-

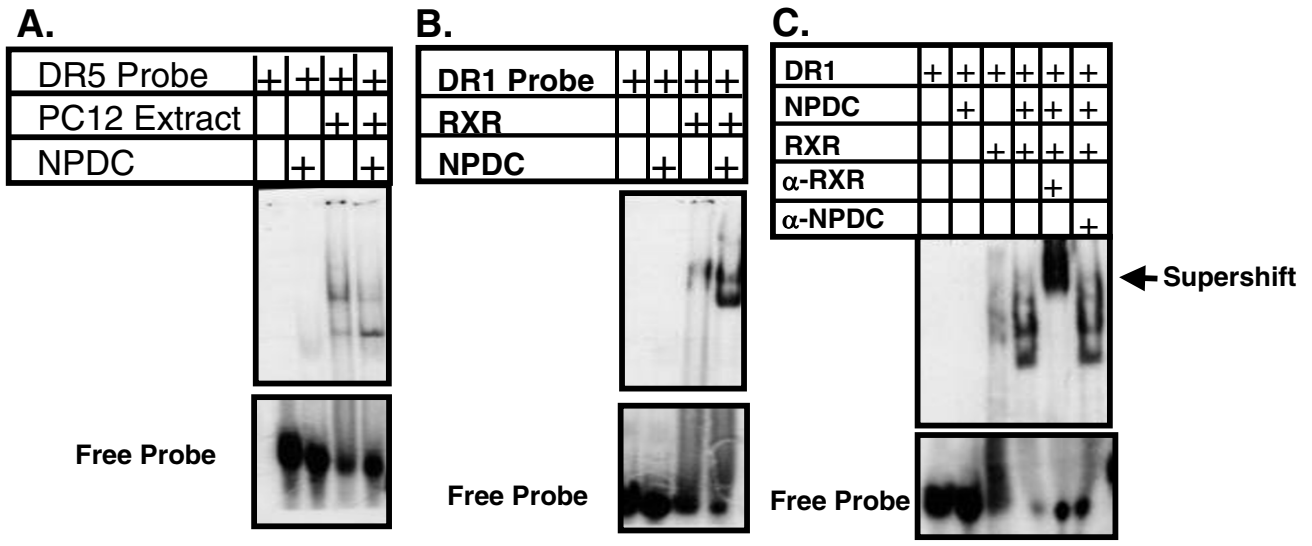

Figure 6
NPDC-1 Alters RARβ••••α and RXRα DNA binding properties. Oligonucleotides corresponding to the consensus RARβ•RARE (DR-5: A) or the consensus RXRα RARE (DR-1: B & C) were end-labeled with ^{32}-P by T4 polynucleotide kinase according to the manufacture's instructions. The resultant DNA binding probes were incubated with PC12 lysate (A), recombinant NPDC-1 and recombinant RXRα (B & C) as indicated. DNA:protein complexes were resolved on non-denaturing PAGE gels which were subsequently exposed to Kodak Xar-5 film. In C, the presence of NPDC-1 within the RXR gel-shift complex was assayed by supershift. Antibodies specific for either NPDC-1 or RXR were used to identify proteins present in the complex.

malian transcription assay for their ability to repress RAR-mediated transcription (Fig. 8). As seen here, none of the mutations were able to impact the repression associated with NPDC-1 cotransfection. These data suggest the LXXLL motifs of NPDC-1 do not play a key role in its ability to repress transcription.

RXR Binds the Amino Terminus of NPDC-1

In the above studies we were able to demonstrate binding of NPDC-1 to retinoid receptors, but the transcription studies suggest this may not be through its LXXLL motifs. To localize NDPC-1 binding to RXR, a series of deletion constructs were made sequentially deleting sequence information from the amino and carboxyl termini of hNPDC-1 (Fig. 9A). These deletion constructs were used to generate recombinant hNPDC-1 protein and were analyzed in standard pull-down assays for their ability to interact with recombinant hRXRα protein (Fig. 9B). As seen in Fig. 9B, deletion of a region between amino acids 76 and 112 substantially reduces NPDC-1 interactions, suggesting the putative coil-coil domain residing in this region may play an important role in NPDC-1 interactions with RXRα.

Discussion

In this study, the gene for the rat homologue of the mouse NPDC-1 protein was identified as a positively regulator of RXR mediated transcriptional activation in yeast. The cloning approach used took advantage of RXR's inherent ability to bind DNA and activate transcription in yeast. By creating the proper conditions for the sensitive detection of minimal transcriptional enhancement, random rat brain cDNA products were screened for their ability to facilitate RXR function. Whereas the yeast two-hybrid system has most often been used to identify a positive protein-protein interaction between cDNA products and the ligand binding domain of nuclear receptors [37–43], the strategy used here screens for a functional relationship between cDNA products and the entire receptor, without the addition of hybrid domains. A rat NPDC-1 expression plasmid autonomously affected transcription of a yeast reporter plasmid when co-expressed with RXR but had no enhancing activity when transformed alone.

The rat NPDC-1 gene sequence was subsequently used to screen a human brain cDNA library from which a human NPDC-1 homologue was identified. Alignment of the

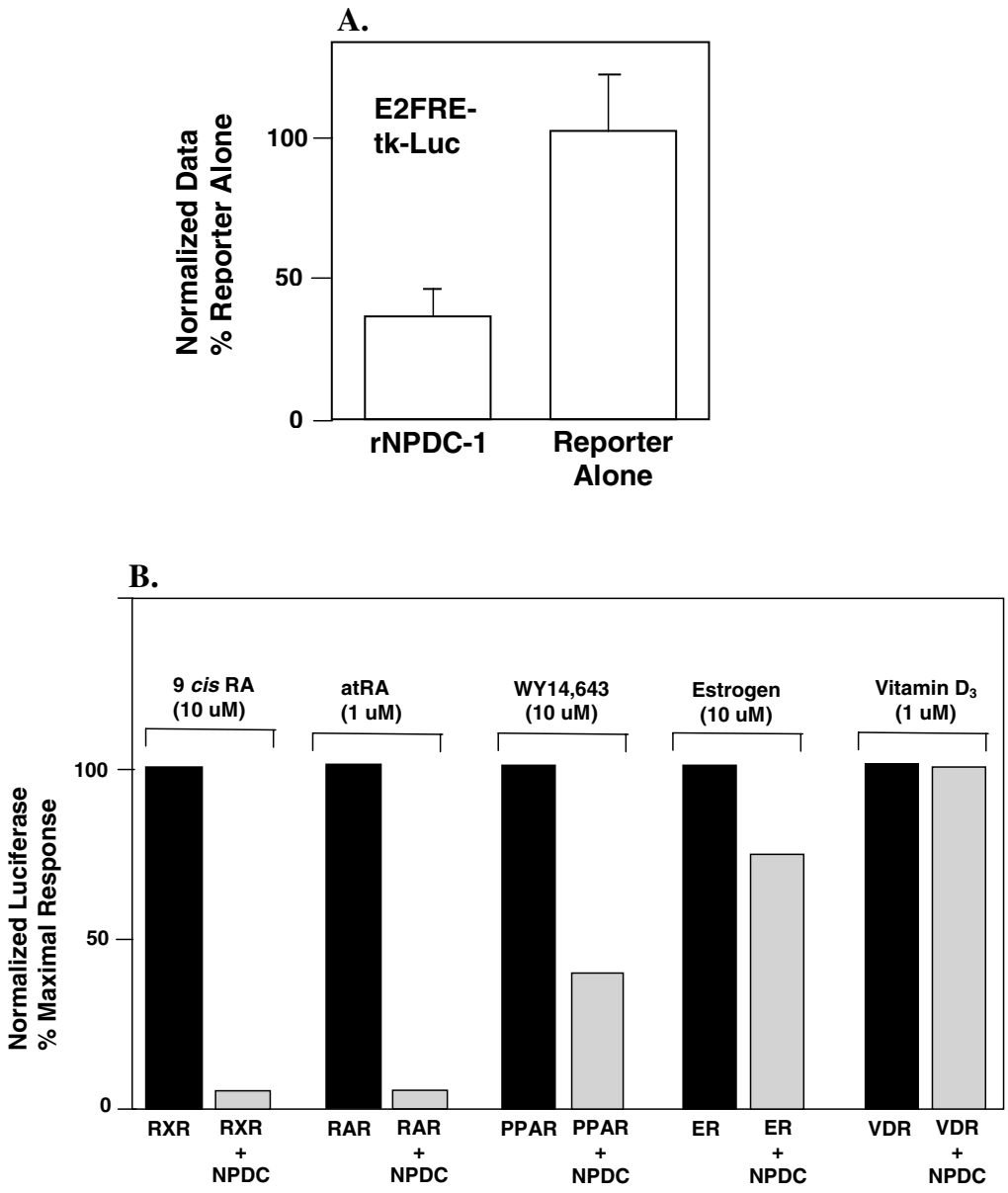

Figure 7

NPDC-1 selectively represses nuclear receptor-mediated transcription. A. rNPDC-1 was analyzed for its ability to modulate the transcription mediated by endogenous E2F transcription factors. A pBL-E2FRE-tk-luc (E2FRE) reporter plasmid and a mammalian expression β-galactosidase construct were transfected into HepG2 cells with and without a mammalian expression construct for rNPDC-1. Luciferase data was normalized as described above and data was reported as a % of the constitutive activation of the E2F reporter plasmid. B. Mammalian expression plasmids for pRS-hRXRα, pRS-hRARβ, pRS-rPPARα, pRS-hERα and pRS-hVDR were transiently transfected into HEK 293 cells along with their respective luciferase reporter constructs {hRXRα: pBL-CRBP2, hRARβ: pBL-βRE(2), rPPARα: pBL-AOXRE, hERα: pBL-ERE, and hVDR: pBL-VDRE(3)} and with or without a mammalian expression construct for rNPDC-1. Transfected cells were incubated with appropriate ligands at indicated doses. A mammalian expression β-galactosidase construct was included in all transfections to normalize for transfection efficiency. Multiple data points were collected and calculated as average induced luciferase activity normalized to constitutive βGal activity {normalized response = average full integral luciferase activity/ [average (βGal activity/ min)]}. Data is expressed here as a percentage of the normalized response for the ligand induced receptor-reporter constructs. Error bars represent the value of standard deviations n = 4.

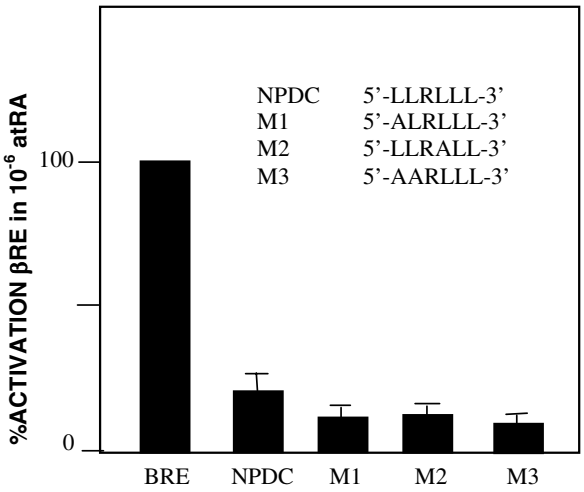

Figure 8
NPDC-1 LXXLL Motifs are not implicated in NPDC-1 corepression of RAR-mediated transcription Site directed mutagenesis was used to change critical leucines in the pCMV-hNPDC-1 LLRLLL motif (see insert in figure for specifics) to alanines. These expression constructs were cotransfected with the RAR/RXR-specific βRE reporter construct into human embryonic kidney cells (HEK) and analyzed for transcriptional activation of the reporter in the presence of 10^{-6} M all trans retinoic acid (atRA). Transfections were normalized to a β-galactosidase expression construct included in all transfections, and data was calculated as described in Fig. 7. Data was expressed as a percent of the reporter alone transfection and statistics were performed on the results of 3 independent experiments. Error bars represent the value of standard deviations n = 3.

mouse NPDC-1 amino acid sequence with the rat and human proteins demonstrated that these proteins are evolutionarily well conserved, indicating that they likely perform a similar and essential function. An LXXLL amino acid motif found in a number of transcription coregulators has been shown to be necessary and sufficient to mediate the binding of coregulators to liganded nuclear receptors [22,23]. X-ray crystal structure analysis suggest the mechanism for the interaction of nuclear receptors with coregulators involves specific contacts between a coregulators LXXLL motif and a highly conserved nuclear receptors motif, termed activating function-2 (AF-2), located at the C terminus of nuclear receptors [44,45]. The three species specific NPDC-1 proteins have a conserved LXXLL amino acid sequence proximal to their amino terminus, but mutation studies presented here suggest this domain does not play an integral role in the repression of transcription mediated by NPDC-1. Furthermore the deletion studies presented here suggest that the NPDC more probably interacts with RXR through an area localized more centrally in the protein. The most obvious protein interaction domain in this region is a coil-coil domain located between amino acids 76–112 [30]. Finally, this observation is not unprecedented in that PNRC, a recently identified nuclear receptor coregulatory protein [46,47], has also been observed to bind nuclear receptors through a motif other than its LXXLL motif. Further studies are ongoing to establish the role of both the NPDC-1 LXXLL motif and coil-coil domain and their functional significance in the coregulation of retinoid receptor-mediated transcription events.

Northern analysis and reverse transcriptase-PCR amplification of RNA showed that the mouse NPDC-1 was preferentially expressed in the nervous system and not detectable in the other tissues examined [27]. In contrast, Northern analysis revealed that human and rat NPDC-1 mRNA is variably, but much more ubiquitously expressed. Similar to previously published data [28], our results suggest that NPDC-1 mRNA in humans exists as multiple splice variant isoforms. The cloned human cDNA isolated was larger than the predominant smaller mRNA species seen in every tissue, indicating that it likely represents a sub-fragment of the larger transcript, although we cannot rule out the existence of a unique homologous mRNA species. In rats we identified a single but abundant brain mRNA species. Its size approximates that found in mouse brain tissue [27] and would be largely represented by our cloned cDNA. Some of the other tissues probed displayed an mRNA species of a similar size along with the variable presence of larger hybridizing transcripts. The tissue specific relative intensities of these larger hybridizing transcripts were not consistent, which confuses their interpretation. Further examination will be required to identify them as splice variant isoforms or as cross hybridizing unique gene products.

Of special note was the seemingly opportunistic cloning of this RXR coregulator in a ligand independent yeast assay. Efficient DNA binding normally requires the ligand dependent homodimerization of RXR [35]. Although RXR mediates significant ligand inducible transcriptional response in yeast, unliganded RXR induces minimal activation from a βRE containing reporter plasmid. RXR has a higher binding affinity for a response element from the cellular retinoic acid binding protein, type II (CRBPII) promoter region [48]. A significant amount of constitutive activity results from a CRBPII response element containing yeast reporter plasmid and unliganded RXR [26], indicating that unliganded RXR is capable of facilitating transcription in yeast and a limiting condition for this

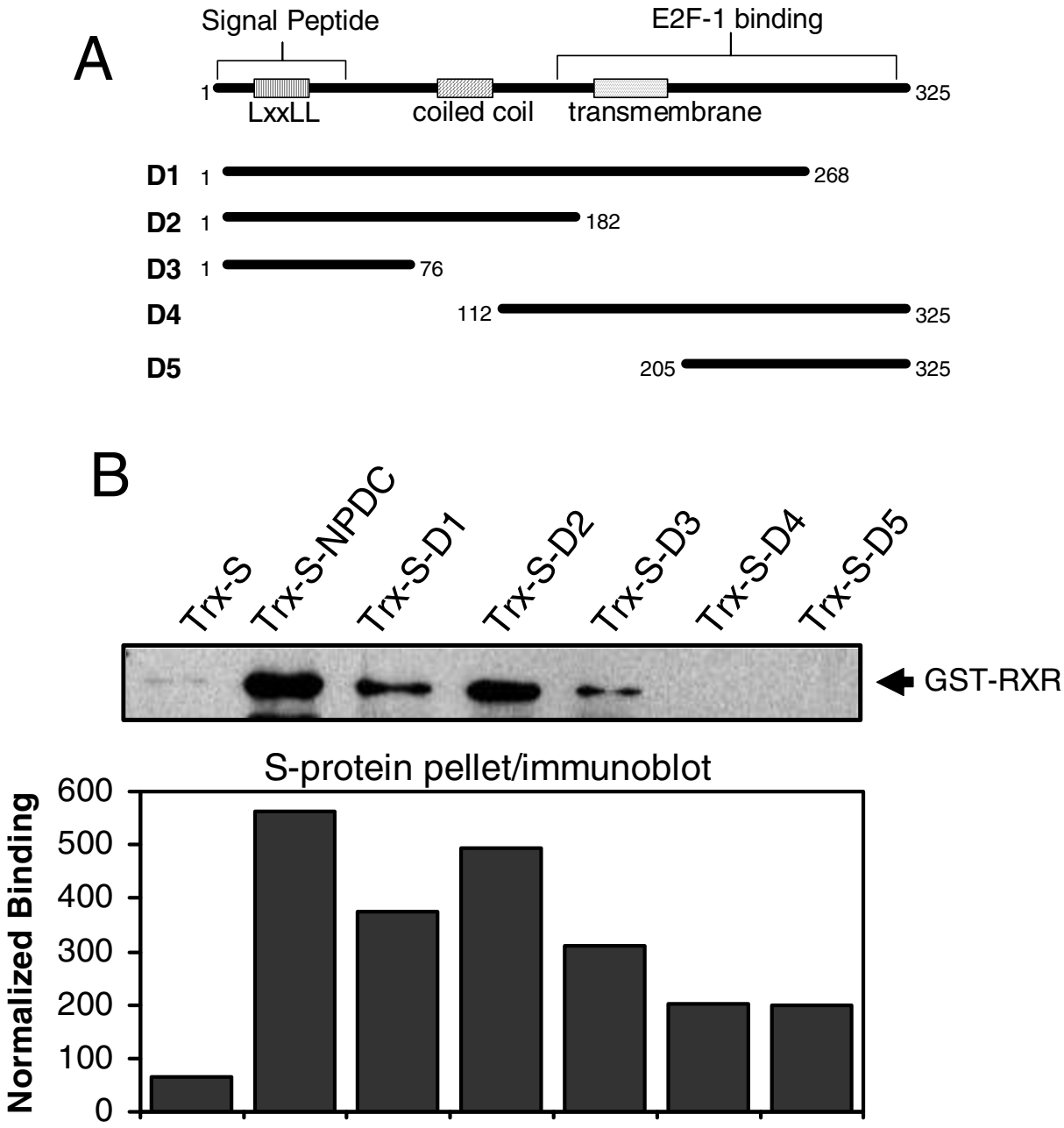

Figure 9

RXR binds the amino terminus of hNPDC-1. Approximately 10 μg of recombinant S-tagged hNPDC-1 or NPDC-1 deletion mutant (A) was pre-coupled to S-protein to generate a 50% slurry of NPDC-1-beads. Reactions containing 30 μl NPDC-beads, 5 μg of recombinant GST-RXRα and 300 μl of 1X gel-shift buffer were incubated at 4°C for 1 hour with rotation. S-protein pellets were collected by centrifugation and washed 3 times in gel-shift buffer. Proteins were released from the pellet by resuspending S-protein agarose in 2X SDS-PAGE sample buffer. The amount of GST-RXRα bound to beads was analyzed by Western blotting onto nitrocellulose filters and probing blots with anti-RXRα (B, top panel). To normalize for unequal expression of the various deletion mutants, duplicate reactions without GST-RXRα were performed and subjected to coomassie staining instead of immunoblotting, and NIH Image Software was used to normalize the bands in the immunoblot (B, top panel) to the amount of S-tagged NPDC-1 precipitated in the coomassie stained gel. Normalized binding is depicted in B, bottom panel.

function may be RXR's ability to bind DNA. From this perspective, it seems likely that the procedure by which we cloned the rat NPDC-1 gene would identify gene products that influence RXR's ability to bind DNA. The gel mobility shift assay results support the contention that NPDC-1 can facilitate the binding of DNA by unliganded RAR/RXR heterodimers or RXR homodimers. Although difficult to establish, there is precedent for an enhancement of DNA binding as a mechanism for nuclear receptor coregulator function. Human homologues of the ***S. cerevisiae*** SWI/SNF family of genes have been shown to function as coregulators of transcriptional activation by nuclear receptors, including RAR [49,50]. These coregulators have been characterized to function as members of large multisubunit complexes that alter chromatin structure to facilitate receptor DNA binding. Human homologues of the ***S. cerevisiae*** SWI/SNF gene products exhibit a protein-protein interaction with the nuclear receptor AF-2 domain and potentially require the receptor's DNA binding ability to associate with specific promoter regions for function [51]. The yeast SWI/SNF complex stimulates the binding of DNA by the yeast transcription factor GAL4 in GMSAs without maintaining a strong association with the protein/DNA complex [52], similar to what is observed here with NPDC-1.

We show that NPDC-1 can repress the induction of transcription in mammalian cells by several nuclear/steroid receptor family members. This activity places NPDC-1 into an expanding list of proteins and RNAs whose structure appear to be targeted to DNA by transcription factors and whose function appears to be integrated into regulation of transcription by the RNA polymerase complex [53–57]. Data demonstrating that NPDC-1 can directly interact with nuclear receptors, can influence nuclear receptor binding to DNA and can bind to and repress E2F mediated transcription events [30] would strongly suggest NPDC-1 is a true corepressor protein. There also appears to be some specificity in NPDC-1's ability to corepress transcription, with very strong corepression occurring with RXR and the RXR heterodimer partner RAR, only moderate transcriptional repression with the RXR heterodimer partner PPAR and the homodimerizing receptor ER, and little if any effects on transcription mediated by the RXR heterodimerizing receptor VDR. However, RXR's promiscuous nature as a heterodimer partner with a number of other receptors implies that NPDC-1 co-regulation of transcription may be dramatic and its expression involved with sweeping changes in the transcriptional regulation of genes within a cell. The up-regulation of mouse NPDC-1 expression has been intimately linked in vivo with a variety of morphological changes as precursor nerve cells begin to terminally differentiate and this expression is maintained up to the adult age [29]. P19 cells are a pluripotent murine embryonic carcinoma cell line that can be induced by atRA to differentiate into cells that are biochemically and morphologically similar to cells of the central nervous system [58]. It has been shown that atRA induced differentiation of P19 cells results in the expression of mNPDC-1 [29]. Whether NPDC-1 expression is directly regulated by an atRA activated RAR or is under the control of some other differentiation induced transcriptional regulator remains to be examined. However, atRA up-regulation of a negative mediator of its own signal would be somewhat inconsistent with this ligand's signaling pathway which is also known to up-regulate the expression of the RARα and RARβ genes [59].

As has been shown for mNPDC-1 [29,30] hNPDC-1 was observed to directly interact with and suppress transcription mediated by E2F-1. E2F trans-activation of transcription is critical for the regulated G1 to S phase transition of the cell cycle [15]. The subsequent down regulation of E2F activity during S phase is also critical for viable cell propagation and is believed to be largely the result of its association with Rb or Rb related proteins [16]. It has been shown that the Rb-E2F complex will bind DNA but function to inhibit E2F-responsive genes. NPDC-1 may inhibit overall E2F transcription by preferentially promoting the binding of the inhibitory complex to DNA over the binding of the activating E2F complex. In the absence of ligand, RAR/RXR heterodimers have been theorized to associate with transcription repressing co-regulators [42,60]. NPDC-1 association with RXR is ligand and RAR heterodimer independent implying that NPDC-1 may also contribute to the repression of RAR responsive genes in the absence of atRA. The E2F-1 factor is thought to play a critical role in the maintenance of cellular proliferation whereas atRA-treated cells normally proliferate at reduced rates. There are some reports suggesting that some intermediate mechanism links these apparently antagonistic pathways [13,61]. Limited availability of NPDC-1 may be involved in the competitive relationship between these transcription factors' function.

Conclusions

The evidence presented here demonstrates that NPDC-1 has remained highly conserved through human and rodent evolution and is capable of binding retinoid receptors, influencing their associations with DNA binding and repressing their ability to regulate gene expression. These results, along with previously published data demonstrating mNPDC-1' s ability to repress proliferation and stimulate differentiation, suggest that NPDC-1 plays an important role in retinoid signaling as cells differentiate. Cumulatively these data categorize NPDC-1 as a tissue-specific corepressor of retinoid-mediated transcription directly linked to differentiation of neuronal cells. Although NPDC-1 has yet to be aligned with any specific neuronal tumorigenesis event it's intrinsic role in

proliferation and differentiation suggest it might function as a tumor suppressor gene. Further studies will be necessary to establish this possibility

Materials and methods

Plasmids and Constructions

The yeast expression plasmids YEphRXRα(TRP), YEphRARγ(LEU) and the yeast transcription reporter plasmids YRpβRE(1) and YRpβRE(2) have been described elsewhere [26]. The mammalian expression plasmid pCMVβ (Clontech) was modified to introduce a bacterial T7 promoter enabling overlapping expression from this promoter and the CMV immediate early gene promoter/enhancer. A polycloning site was also introduced downstream from these promoters, replacing the β-galactosidase (βGal) gene, and the resulting plasmid was designated pCMV/T7PC. ***NotI*** fragments containing the cloned rat and human NPDC-1 genes were sub-cloned into pCMV/T7PC creating pCMVrNPDC-1. The mammalian expression plasmids pRShRARβ•[26], pRShRXRα• [26], pRShERα [62], pRSrPPARα•[25], pRShVDR [63] and pRSβGal [62] are described elsewhere. The mammalian reporter plasmids βRE-tk-luc and E2FRE-tk-luc were constructed by sub-cloning a single copy of annealed synthetic oligonucleotides into ***HindIII*** or ***SalI*** – ***BamHI***, respectively, prepared pBL-tk-luc vector [62]. The sequence of the oligonucleotides used were as follows: βRE, 5'-AGCTTAAGGGTTCACCGAAAGTTCACTCGCAT-3' and 3'-ATTCCCAAGTGGCTTTCAAGTGAGCGTATCGA-5'; E2FRE, 5'-TCGACATGCAATTTCGCGCCAAACTTG-GGA-3' and 3'-GTACGTTAAAGCGCGGTTTGAACCCTC-TAG-5'. The mammalian reporter plasmid ERE(5)-MTV-luc was constructed by sub-cloning five copies of annealed synthetic oligonucleotides (5'-AGCTGTCAGGTCACAGT-GACCTGAT-3' and 3'-CAGTCCAGTGTCACTGGAC-TATCGA-5') into ***HindIII*** prepared MTV-luc [64].

Single amino acid mutations in the LLRLLL motif of the pCMV-hNPDC-1 cDNA were made using the QuikChange Site Directed Mutagenesis Kit (Stratagene) and protocols as described in the users manual. Oligonucleotides used in the construction of the various mutations include M1-ALRLLL

(5'-CGCGGCACCTGCGG**gc**GCTGCGGCTGCTGCTCTC-CGGCCTCGTCC-3' and

5'-GGACGAGGCCGGAGAGCAGCAGCCGCAGCGCCCGC AGGTGCCGCG-3'), M2-LLRLAL (5'-CGCGGCACCTGC-GGCTGCTGCGGCTG**gc**GCTCTCCGGCCTCGTCC-3' and

5'-GGACGAGGCCGGAGAGCGCCAGCCGCAGCGCCC-GCAGGTGCCGCG-3') and M3-AARLLL

5'-CGCGGCACCTGCGG**gc**G**gc**GCGGCTGCTGCTCTCC-GGCCTCGTCC-3' and

5'-GGACGAGGCCGGAGAGCAGCAGCCGCGCCGCCC-GCAGGTGCCGCG-3'). The integrity of introduced mutations was verified by DNA sequencing.

A bacterial expression construct for full-length hNPDC-1 and a series of hNPDC-1 mutant constructs were generated in pET-32a (Novagen). Synthetic oligonucleotide primers for full-length hNPDC-1 (forward: 5'-TTGAAT-TCATGGCGACGCCGCTGC-3' and reverse: 5'-TTGGATC-CAGTGGCAGTGCAGGCGGTG-3'), a D1 mutant containing amino acids 1–268 (forward: 5'-TTGAAT-TCATGGCGACGCCGCTGC-3' and reverse: 5'-TTGGATC-CATGGGTGGCTCTTTATGC-3'), a D2 mutant truncating the predicted transmembrane domain of hNPDC-1 and containing amino acids 1–182 (forward: 5'-TGAATTCAT-GGCGACGCCGCTGC-3' and reverse: 5'-TTGGATCCAT-GCCGTCGCCTTGCCCTC-3'), a D3 mutant truncating NPDC-1 just before the putative coil-coil domain and containing amino acids 1–76 (forward: 5'-TTGAATTCAT-GGCGACGCCGCTGC-3' and reverse: 5'-TTGGATCCATC-CCTTGCTGGTCCTCC-3'), a D4 mutant retaining the putative transmembrane domain but deleting the LXXLL motif and containing residues 112–325 (forward: 5'-TTGAATTCGGACACTCAACTCCGC-3') and reverse: 5'-TTGGATCCAGTGGCAGTGCAGGCGGTG-3') and a D5 mutant retaining all residues after the putative transmembrane domain and containing residues 205–325 (forward: 5'-TTGAATTCTGCTGGTGCAGGCTGCAG-3' and reverse: 5'-TTGGATCCAGTGGCAGTGCAGGCGGTG-3') were used in polymerase chain reaction technology (PCR) reactions to generate a full-length hNPDC-1 and mutant fragments containing terminal ***EcoRI*** (5') and ***BamHI*** (3') restriction sites. Subsequently the hNPDC-1 and hNPDC-1 mutant PCR fragments were subcloned into ***EcoRI***/***BamHI*** restricted pET32-a vector. These constructs generated hNPDC-1 proteins tagged with thioredoxin (TRX), 6xHis, and S-protein at its amino terminus. The integrity of all plasmid constructs and the composition of unique cloned cDNAs were confirmed by dideoxy sequencing.

Yeast Expression Library Screening and Transcription Assay

Reconstitution of RXR transcriptional activation in the auxotrophic mutant ***S. cerevisiae*** cell line BJ5409 (MAT•, ***leu***2D, ***his***3D200, ***ura***3–52, ***tr***1) has been described elsewhere [26]. An established YEpRXRα (TRP), YRpβRE(1) transformant was further transformed with a rat brain cDNA yeast expression library [31], a generous gift of Dr. Robert Dickson, University of Kentucky, by the method of lithium acetate [65]. Transformed yeast were plated onto yeast nitrogen base, 2% bacto agar plates supplemented with 2% glucose, 20 μg/ml adenine sulfate, 20 μg/ml his-

tidine-HCl, 10 μM CuSO4, 0.5 μM all-trans retinoic acid and 0.06 mg/ml 5-bromo-4-chloro-3-indolyl-β-***o***-galactopyranoside (X-GAL). Activation of βGal transcription by yeast grown in solution was determined as previously described [26] with the following modifications. The βGal enzymatic activity present in yeast extracts after 22–24 hr incubation in minimal media plus 25 μM CuSO4 was determined by measuring the conversion of the ***o***-nitrophenyl-β-D-galactopyranoside (ONPG) colorimetric substrate (A_{415}) normalized to cell number approximations (A_{600}). Yeast ONPG transcription assays were repeated at least twice with separate transformants and yielded similar results.

Cloning human NPDC-1

A full-length cDNA fragment for rNPDC-1 was labeled with ^{32}P by nick translation and used to screen a λgt-11 human fetal brain cDNA library (Clontech) [66]. Utilizing dideoxy DNA sequencing techniques, a 2.5 kb insert containing λgt-11 clone was established to harbor the complete coding sequence for the human homolog of mNPDC-1 [28].

Northern Blot Analysis

Rat and human Multiple Tissue Northern (MTN) blots were purchased from Clontech Laboratories Inc. The rat and human cDNA clones were used to prepare [α-^{32}P]-labeled DNA probes by nick translation of double-stranded fragments [66]. The blots were probed using ExpressHyb solution as suggested by the manufacturer (Clontech).

Bacterial Protein Expression

Bacterially expressed NPDC-1 (Trx-S-NPDC) was purified from BL21-CodonPlus-RP E. coli (Stratagene) harboring the pET32a-hNPDC-1 expression construct. An overnight culture was diluted 1:5 into 2xYT bacterial media and incubated at 37°C for 2 hours. Subsequently, cells were moved to room temperature, grown to 0.6 OD600 and induced with 1 mM IPTG for an additional 4 hours. Cells were collected at 6000 × g for 10 minutes. The pellet was resuspended in 20 ml triple detergent lysis buffer (50 mM Tris-8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, and 0.5% sodium deoxycholate) supplemented with 1 mg/ml lysozyme, protease inhibitors (Roche) and Benzonase nuclease (Novagen). Cells were allowed to lyse for 20 minutes at room temperature. Lysate was clarified in 50 ml Oakridge tubes at 25,000 × g for 20 minutes. Clarified lysate was injected onto HiTrap chelating HP columns (Amersham Pharmacia) preloaded with nickel. Bound protein was washed with 10 column volumes of His-wash (50 mM Tris-8.0, 500 mM NaCl, and 20 mM Imidazole). Proteins were eluted in His-elution buffer (50 mM Tris-8.0, 500 mM NaCl, and 500 mM Imidazole) and subsequently desalted on a HiTrap desalting column (Amersham Pharmacia) into TBS-P (10 mM Tris-7.6, 150 mM NaCl, and 10% glycerol).

Bacterially expressed pGEX-hRXRα was purified using standard GST-purification techniques except as modified below. Cells were collected and clarified lysates were produced as above. Lysates were injected onto a GST-rap HP column (Amersham Pharmacia) and washed with 10 column volumes of TBS-P. Proteins were eluted with 25 mM glutathione pH 7.0 and desalted into TBS-P.

Gel Mobility Shift Assay

Gel mobility shift assays were performed essentially as described previously [11]. Briefly, annealed synthetic oligonucleotides coding for the DR-5 RARE (5'-AGCTTCAGGTCAGAAGGTCAGAGAGCT-3') and DR-1 RARE (5'-TCGAGGGTAGGGTTCACCGAAAGTTCACTCG-3') were radioactively end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (PNK) [66]. Labeled probes were incubated with variable combinations of recombinant protein or PC12 nuclear extract in a 20 ul reaction mixture (5 mM Tris pH 7.9, 15 mM HEPES-KOH pH 7.9, 5 mM EDTA, 3.5 mM MgCl2, 5 mM DTT, 0.1% Tween-20, 10% Glycerol, 50 mM KCl) as follows: NPDC (10 μg) and RXR (5 μg) plus PC12 (25 μg). The protein/DNA complexes were separated from free radiolabeled DNA by electrophoresis in non-denaturing polyacrylamide gels and autoradiographed. In order to identify proteins present in complexes, supershifts with antibodies to RXRα (D-20, Santa Cruz) and NPDC-1 (custom antibody and α-thioredoxin, Thio-probe, Santa Cruz) were used with the above reactions.

In Vivo Pull-downs

The ability of NPDC to interact with in vivo expressed RARβ and RXRα was assayed by using Trx-S-NPDC as a pull-down reagent. PC12 cells were transfected with either pCMV-RARβ or pCMV-RXRα using Effectene reagent (Qiagen). After 48 hours cells were lysed on the plate in 200 μL of lysis buffer-PD (50 mM Tris-7.5, 150 mM NaCl, 20 mM $MgCl_2$, 10% glycerol, 1% NP-40) supplemented with 1X protease inhibitors (Calbiochem).

Trx-S-NPDC was prebound to S-protein covalently attached to agarose beads for 20 minutes. 30 μL of a 50% slurry of beads was added to 300 μL of buffer PD. 100 μg of cell lysate expressing HA-E2F-1, RARβ, or RXRα were added to the tubes and incubated with rotation for 1 hour at 4°C. Beads were collected and washed three times in buffer PD. Bound-proteins were eluted by adding 20 μL of 2X SDS-PAGE sample buffer. Samples were run on a 12.5% SDS-Page gel and immunoblotted onto nitrocellulose filters. Filters were probed with anti-HA (Cell Signaling), anti-RARβ (C-19, Santa Cruz), or anti-RXRα (D-20, Santa Cruz).

In Vitro Pull-Down Assays

Agarose-glutathione (Ag-G) bound Glutathione-S-Transferase (GST) and GST-RXR fusion protein were prepared from BL21 (DE3) LysS cell transformants as described above, with the exception that agarose bound recombinant protein was not eluted but rather stored bound to beads. Bacterial extracts containing pETCFlag, pETrNPDC(F) and pEThRARγ were generated as described above with the exception that cleared soluble fractions were used or stored at -70°C without further affinity purification. Protein concentrations were determined using Bio-Rad Protein Assay per the manufacturer's instructions (Bio-Rad).

The pull-down assays were performed as described previously [67]. Briefly, 30 μl of a GST-RXR agarose bound 33% protein slurry was pre-equilibrated with NENT buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) and incubated for 60 min at room temperature with 40 μg of pETrNPDC(F) extracts without and/or with 60 μg of pEThRARγ extracts or 1 μM 9-cis retinoic acid. Successive washes with NENT removed unbound material and bound material was eluted from Ag-G by incubation in electrophoresis sample buffer. Eluted proteins were Western blotted onto nitrocellulose filters and probed with an anti-Flag M2 monoclonal antibody (Sigma) at 30 μg/ml. Immunoreactive bands were detected by autoradiography on X-ray film. MultiMark pre-stained, multi-colored proteins (NOVEX) were used to assess protein transfer and as molecular weight standards.

Mammalian Cell Transcription Assay

Transient transfections were conducted with either the human embryonal kidney cell line 293, or the human hepatocellular carcinoma cell line, HepG2. The cells were transiently transfected by the method of calcium phosphate as previously described [68]. Following a 6 hr incubation with transfecting plasmids in 10 cm plates, cells were replated into 96 well plates, plus or minus activating ligands. After 42–48 hours, induced luciferase and normalizing βGal activities were determined as previously described [69]. The amount of plasmid used per 10 cm plate transfection was as follows: βRE-tk-luc, 2 μg; ERE(5)-MTV-luc, 4 μg; E2FRE-tk-luc, 5 μg; pRShER, 1 μg; pCMVrNPDC-1 or pCMVhNPDC-1, 4 μg in βRE and E2FRE experiments and 2 μg in ERE experiments; pRSβGal, 4 μg in βRE and ERE experiments and 5 μg in E2FRE experiments. The total amount of plasmid per 10 cm plate transient transfection was brought to 20 μg with pUC19 DNA.

Abbreviations

RXR, retinoid-X receptor; RAR, retinoic acid receptor; atRA, all-trans retinoic acid; 9cRA, 9-cis retinoic acid; HRE, hormone response element; βRE, RAR beta response element; DR(n), direct repeat of the hormone response element consensus sequence separated by "n" basepairs; NPDC-1, neural proliferation differentiation and control protein one; Rb, retinoblastoma; ORF, open reading frame; βGal, beta-galactosidase; pcRB, plasmid expressing rat brain cDNA; ONPG, ***o***-nitrophenyl-β-D-galactopyranoside; X-GAL, 5-bromo-4-chloro-3-indolyl-β-***o***-galactopyranoside; CMV, cytomegalovirus; GST, glutathione-S-transferase; Ag-G, agarose bound glutathione; GMSA, gel mobility shift assay; ERα, estrogen receptor alpha; ERE, estrogen receptor response element; E2FRE, E2F response element; E_2, beta estradiol; AF-2, activating function-2; CRBPII, cellular retinoic acid binding protein, type II; MTN, multiple tissue Northern.

Competing Interests

None declared.

Authors Contributions

KWH was involved in the conceptualization of these studies, carried out the yeast cloning studies that resulted in the isolation of NPDC and participated in all of its initial characterizations. MLS participated in the experiments demonstrating NPDC binding to retinoid receptor, the localization of that binding on the receptor as well as its association with E2F. MT participated in the experiments demonstrating NPDC-1 regulation of retinoid receptor binding to DNA. DL participated in the mutational analysis of the hNPDC-1 LXXLL motif. DJN was involved in the conceptualization and direction of these studies, participated in experiments demonstrating NPDC-1's function as a coregulator of steroid receptor-mediated transcription as well as the writing of the manuscript. All authors read and approved the final manuscript.

Acknowledegements

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